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Therapeutic PD-1 Pathway Blockade Augments with other Modalities of Immunotherapy to Prevent Immune Decline in Ovarian Cancer

Jaikumar Duraiswamy¹, Gordon J. Freeman², and George Coukos^{1,3,*}

¹Ovarian Cancer Research Center, Perelman School of Medicine, University of Pennsylvania, PA, USA ²Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA

³Department of Oncology and Ludwig Center for Cancer Research, University Hospital of Lausanne, Switzerland

Abstract

The tumor microenvironment mediates induction of the immunosuppressive programmed death-1 (PD-1) pathway, targeted interventions against which can help restore antitumor immunity. To gain insight into these responses, we studied the interaction between PD-1 expressed on T cells and its ligands (PD-1:PD-L1, PD-1:PD-L2, and PD-L1:B7.1) expressed on other cells in the tumor microenvironment, using a syngeneic orthotopic mouse model of epithelial ovarian cancer (ID8). Exhaustion of tumor-infiltrating lymphocytes (TILs) correlated with expression of PD-1 ligands by tumor cells and tumor-derived myeloid cells, including macrophages (TAM), dendritic cells (DC) and myeloid-derived suppressor cells (MDSC). When combined with GVAX or FVAX vaccination (consisting of irradiated ID8 cells expressing GM-CSF or FLT3 ligand) and co-stimulation by agonistic α 4-1BB or TLR 9 ligand, antibody mediated blockade of PD-1 or PD-L1 triggered rejection of ID8 tumors in 75% of tumor-bearing mice. This therapeutic effect was associated with increased proliferation and function of tumor antigen-specific effector CD8⁺ T cells, inhibition of suppressive T regulatory cells (Tregs) and MDSC, upregulation of effector T cell signaling molecules and generation of T memory precursor cells. Overall, PD-1/PD-L1 blockade enhanced the amplitude of tumor immunity by reprogramming suppressive and stimulatory signals that yielded more powerful cancer control.

Introduction

At the time of diagnosis, over 75% of patients with ovarian cancer present with advanced stage III or IV disease (1–2). Despite appropriate surgery and receiving highly effective first-line chemotherapy, ~70% of patients with advanced-stage disease who achieve remission eventually relapse (1–2). Thus, there is an immediate need for therapeutic targets for treating ovarian cancer (3). Our group and others have reported that tumor-infiltrating T lymphocytes (TILs) with anti-tumor potential exist in cancer patients (4–7). Studies in a primary co-culture system showed that TILs from many ovarian cancer patients secrete low to intermediate levels of IFN- γ and limited proliferation in response to cognate peptides (unpublished observation). The programmed cell death 1 (PD-1) is an inhibitory surface receptor expressed by T cells, B cells, natural killer T cells, monocytes, and DCs, but not by resting T cells. PD-1 binds two ligands, programmed cell death ligand 1 (PD-L1) and PD-

*Correspondence: **Dr. George Coukos MD, PhD**, george.coukos@chuv.ch.

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L2, also called B7-H1 and B7-DC, respectively (8–9). Tumors can employ the PD-1 inhibitory pathway to silence the immune system (8). The expression of PD-L1 in tumors is inversely correlated with survival of patients (10–11). This indicates that although anti-tumor immunity is elicited against ovarian cancer, it is counterbalanced by immunosuppressive factors.

In ovarian tumors, myeloid cells are one of the major determinants of immune suppression. These include tumor-associated macrophages (TAMs), immature/tolerogenic DCs, and myeloid-derived suppressor cells (MDSCs) (12–21). In addition, CD4⁺CD25⁺Foxp3⁺ T regulatory cells (Tregs) play a critical role in the control of anti-tumor immune responses, relying on PD-1, PD-L1 or cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) to perform these functions (22–27). Most studies describe mechanisms for the accumulation of these immunosuppressive myeloid cells or Tregs. In this study, we demonstrate a cross-regulation among these cell types using the ID8 syngeneic mouse model of epithelial ovarian cancer. We provide evidence that T cell dysfunction can be reversed by targeting the PD-1 pathway simultaneously in all these cell types. We found that expansion of ovarian antigen-specific CD8⁺ TILs was dependent on the amount of PD-L1 signaling by tumor cells, tumor-derived myeloid cells and Tregs. Furthermore, combining PD-1 blockade with a single dose of GVAX or FVAX vaccination resulted in enhanced clonal expansion of antigen-specific CD8⁺ T cells and tumor control. Finally, we observed a further boost of CD8⁺ T cell function when PD-L1 blockade was combined with both vaccination and α 4-1BB co-stimulation. Overall, our study shows that α PD-L1 blockade therapy greatly synergizes with other immunotherapy modalities.

Methods

Mice and tumor lines

All experiments were performed using protocols approved by the University of Pennsylvania Laboratory Animal Resources (ULAR) policies. A mouse ovarian epithelial papillary serous adenocarcinoma cell line (ID8) was obtained from Dr. K. F. Roby, University of Kansas Medical Center (28). Development of ID8 cells expressing murine GM-CSF (ID8-GVAX) or Flt3-ligand (ID8-FVAX) was based on methods described previously (29).

Blocking and agonistic antibodies

Rat anti-mouse PD-1 (29F.1A12, at IgG2a, *k*), PD-L1 (10F.9G2, rat IgG2b, *k*), PD-L2 (3.2, mouse IgG1), and isotype control antibodies were used (7). The rat anti-mouse 4-1BB antibody from BioXcell was also used.

Tumor experiments

C57BL6 mice (n=12) were given an intraperitoneal (i.p) injection containing 5×10^6 ID8 cells. Two hundred micrograms of rat α -mouse PD-1, -PD-L1, -PD-L2 antibodies, 100 μ g of α -CTLA-4 (clone 9D9) or isotype control antibodies were administered i.p. 4 weeks after tumor inoculation 5 times on alternate days. For co-stimulation 200 μ g of α 4-1BB antibody (BioXcell) was given once along with first aforementioned dose of blocking antibodies. In vaccination experiments, 10^6 irradiated (150Gy) gene-modified ID8-GVAX or ID8-FVAX cells were given by i.p. once, 3 weeks after tumor inoculation. Mice were monitored by their weight gain twice a week. Mice weighing >35 gm as a result of tumor growth and/or ascites fluid accumulation were euthanized.

Methods are detailed in the supplementary section.

Results

Lack of T cell infiltration in ovarian tumors is acquired

We have previously reported that, at the steady state, established ID8 tumors are poorly infiltrated by T cells (33) and are thus considered intrinsically non-immunogenic, mimicking many human ovarian cancers lacking TILs (6). In this orthotopic model of ovarian cancer, gross metastatic i.p. nodules appear at approximately 28 days, and tumor and ascites rapidly accumulate leading to 100% death at 55–60 days (Fig. 1a). We investigated the changes in the frequency of TILs in ovarian tumors, ascites, and spleens of mice from early (~28 days) to late times of tumor growth (~52 days). Interestingly, CD3⁺ (CD8⁺ and CD4⁺) T cells infiltrating during the early tumors were almost completely absent in advanced tumors (Fig. 1b).

Interestingly, a high percent of T cells in advanced tumors were Tregs (34), while there were no detectable CD8⁺ T cells left and the depletion of T cells was restricted to tumors. Parallel to the observed disappearance of CD3⁺ TILs and the increase in Treg cells, we found a significant increase in MDSCs (CD11b⁺Gr-1⁺, $p=0.024$) (Fig. 1b; Supplementary Fig. 1) and TAMs (CD11b⁺F4/80⁺, $p=0.048$) in tumors, but no significant changes in CD11c^{hi} DCs ($p=0.10$) (not shown). Thus, lack of immunogenicity in established ID8 tumors appears to be acquired rather than intrinsic.

PD-1 ligands are present in the microenvironment of ovarian tumors

Previous immunohistochemical studies have reported upregulation of PD-L1 in human ovarian cancers (10). Here we observed high levels of PD-L1 and moderate levels of PD-L2 on ID8 tumor cells, as well as macrophages, DCs and MDSCs derived from the same tumors (Fig. 2a–b; Supplementary Fig. 2a). Similarly, myeloid cells derived from ascites showed moderate expression of high levels of PD-L1 and PD-L2, however, spleens showed only low level expression of PD-L1, but not PD-L2. Next, we found that PD-1 was expressed by matched CD8⁺ T cells in tumor at both early and later stages (Fig. 2c; Supplementary Fig. 2b), but PD-1 expression was not detected in other organs except PD-1^{dim} CD8⁺ T cells in the liver at early stages of tumor. Thus T cells specifically upregulate PD-1 in the tumor microenvironment where they are likely to receive inhibitory signals.

PD-1 or PD-L1, but not PD-L2, blockade therapy cause regression of ovarian tumors

We next tested whether the blockade of PD-1 could reverse the decline of T cell immunity and promote tumor rejection in mice. We also sought to compare the relative contribution of PD-L1 *versus* PD-L2. We inoculated C57BL/6 mice i.p. with ID8 tumor cells and then administered α -PD-1, α -PD-L1 or α -PD-L2 antibodies starting on day 28 (Fig. 3a **left**). Treatment with α -PD-1 or α -PD-L1 antibodies resulted in tumor rejection in 25% (3/12) of the mice, as indicated by normalized mouse weights after treatment (weight gain is due to ascites fluid accumulation and is an accurate surrogate of tumor growth in this model), and long-term survival. On the contrary, α -PD-L2 antibody did not reject tumors. To test whether PD-1-mediated TIL suppression was active at even earlier stages of tumor development, we treated mice with α -PD-L1 starting on day 21. PD-L1 blockade resulted in tumor rejection in 60% (7/12) of the mice (Fig. 3a **right**). Thus, the PD-1 pathway is highly relevant, and is active very early in the process of establishment of ovarian tumors.

PD-1 or PD-L1, but not PD-L2, blockade therapy prevent immune decline in the tumor microenvironment

Next, we tested whether PD-1 blockade was associated with reversal of immune decline in tumors and enhanced TIL infiltration. We chose the more advanced treatment schedule

(starting on day 28), as above. A week after completion of the treatment (day ~45), residual peritoneal tumor deposits were resected and analyzed. The frequency of CD8⁺ and CD4⁺ T cells (as well as total CD45⁺ leukocytes) was markedly increased following α -PD-1 and α -PD-L1 administration. Interestingly, in spite of the lack of tumor response, there was an increase in CD8⁺ and total CD45⁺ cells also following α -PD-L2 (Fig. 4a). Importantly, PD-1 and PD-L1 blockade significantly reduced Tregs and thereby increased the CD8⁺ to Treg and CD4⁺ to Treg cell ratios within the tumor (Fig. 4b). On the contrary, no such decrease in Tregs was seen after α -PD-L2 treatment, and thus there was no significant change in the CD8⁺ or CD4⁺ to Treg ratios.

We also noticed PD-L1 surface expression on Tregs (Supplementary Fig. 1). To understand whether PD-1-PD-L1 interactions contribute directly to the inhibitory function of tumor Tregs, CD4⁺CD25⁺ Tregs isolated from ID8 tumors were incubated with responder spleen CD8⁺ T cells stimulated by immobilized α -CD3 antibody, in the presence or absence of PD-L1 blocking antibody. T cell proliferation was assessed by CFSE dilution. Whereas Tregs suppressed responder cell proliferation, α -PD-L1 attenuated the ability of Tregs to suppress CD8⁺ T cell proliferation (Fig. 4c, $p < 0.05$; Supplementary Fig. 3). Thus, PD-L1 blockade not only reduced the relative frequency of Tregs in ID8 tumors, but also attenuates the suppressive function of tumor-derived Tregs.

MDSCs were also decreased specifically by PD-L1 blockade, which thereby increased the ratio of T cells to MDSCs within the tumor (Fig. 4d). MDSCs suppress effector T cells through the production of arginase-I (40, 44). Interestingly, we detected a significantly lower number of arginase-I positive MDSC in ID8 tumors after treatment with α -PD-L1 (Fig. 4e). To assess whether arginase-I is involved in the mechanisms through which PD-L1⁺ MDSCs suppress TILs in ID8 tumors, CD11b⁺ Gr1⁺ isolated from ID8 tumors were further cultured with ID8 tumor cell supernatants in the presence or absence of α -PD-L1 antibody. Arginase catalyzes the conversion of arginine to ornithine and urea in the urea cycle. Arginase activity was evaluated by measuring the urea concentration in the media. We found that PD-L1 blockade decreased the level and activity of arginase-I (Fig. 4f; $p = 0.0003$). Thus, PD-L1 blockade not only reduced the relative frequency of CD11b⁺ Gr1⁺ cells, but also reduced their suppressive phenotype by reducing arginase-I activity, which appears to be dependent in part on PD-1-PD-L1 interactions.

The above data indicate that PD-1 or PD-L1 blockade significantly attenuated the number and function of Tregs and/or MDSC. We thus tested whether the above changes were associated with activation of CD8⁺ TILs. We detected significant upregulation of Ki-67 and intracellular granzyme B, as well increased levels of phosphorylated transcription factors, T-bet, Eomes and S6 kinase, and Akt, supporting the notion that effective PD-1 blockade activated function and survival pathways in CD8⁺ TILs (35–36) (Fig. 4g; Supplementary Fig. 4).

Vaccination synergizes with PD-1 blockade to prevent immune decline in tumors

We theorized that vaccine administered in combination with PD-1 blockade could enhance the efficacy of PD-1 blockade in preventing immune decline in tumors. Previous studies have described a therapeutic efficacy of B16 autologous melanoma tumor vaccines expressing either granulocyte macrophage colony-stimulating factor (GM-CSF) or FMS like tyrosine kinase 3 ligand (Flt3-ligand), when combined with the blocking CTLA-4 (29, 37–40). We transduced ID8 whole tumor cells to express either GM-CSF (ID8-GVAX, Fig. 5a) or Flt3L (ID8-FVAX, Fig. 5b) and used radiated cells as vaccine in combination with PD-1, PD-L1, or PD-L2 blockade. Mice received weekly vaccine, starting 3 weeks after tumor inoculation. A week later, mice were treated with PD-1 blocking antibodies 5 times on alternate days. Although GVAX alone had no effect on tumor growth or mouse survival,

addition of GVAX doubled the effect of checkpoint blockade on mouse survival. Fifty percent of mice that received GVAX plus PD-1 or PD-L1 blockade rejected their tumors, while antibodies alone resulted in 25% tumor rejection (Fig. 5a **left**). By contrast, α -PD-L2 did not show any positive interaction with vaccination. Similar results were obtained with FVAX (Fig. 5b **left**). Interestingly, the positive interaction between vaccine and checkpoint blockade required full development of the vaccine effect, and this was achieved by increasing the time interval between vaccination and checkpoint blockade to one week (data not shown).

Co-stimulatory signals further augment the combination of vaccine plus PD-L1 blockade

The above results indicate that PD-1 or PD-L1 blockade can prevent immune decline in tumors and that additional of vaccine further enhances this effect. Next we hypothesized that adding a costimulatory signal, 4-1BB (CD137), a tumor necrosis factor receptor gene family member (41–45), could further boost TILs and enhance the effects of checkpoint blockade and vaccination. Mice were vaccinated with GVAX or FVAX starting 3 weeks after tumor inoculation, and a week later, we administered 5 doses of α PD-L1 on alternate days plus a single dose of α 4-1BB (200 μ g) antibody with the first dose of α PD-L1 (Fig. 5a **right**). Administration of the α 4-1BB antibody alone had no impact on tumor growth or survival. Furthermore, addition of α 4-1BB antibody to PD-L1 blockade had moderate impact on survival (Fig. 5a **right**). Remarkably, 75% of mice receiving GVAX followed by α PD-L1 plus α 4-1BB antibodies rejected their tumors. Similar results were obtained with FVAX (Fig. 5b **right**).

In tumors, costimulatory signals could be provided by properly activated antigen-presenting cells, which express the 4-1BB ligand as well as other important costimulatory ligands. Hypothesizing that similar therapeutic benefit as above could be obtained through T cell costimulation by TLR-activated APCs, we used an agonistic murine TLR9 ligand, CpG 1668 (46). Mice were treated with 5 doses of CpG 1668 i.p. (5 μ g/mouse) in combination with α PD-L1 antibody (Supplementary Fig. 5). Similar to α 4-1BB, we found significant therapeutic benefit by adding CpG. Importantly, the effect from this combination was seen only when CpG was given in combination with α PD-L1 antibody but not if CpG was given earlier, in combination with the vaccine, indicating that CpG did not contribute at enhancing T cell priming. Thus, the therapeutic PD-L1 blockade has the potential to induce significant tumor rejection, which can be maximized if PD-L1 blockade is preceded by tumor vaccination, and accompanied by costimulation.

Combination therapy reprograms the tumor immune microenvironment

We next asked what were the changes in the tumor microenvironment induced by the ineffective vaccines GVAX and FVAX. Interestingly, vaccine alone increased significantly the frequency of TILs, especially CD8⁺ cells ($p < 0.01$) (Fig. 5a and Supplementary Fig. 7a). Because vaccines proved ineffective, we asked whether this was associated with an increase in immunosuppressive populations. Importantly, we noticed that both GVAX and FVAX failed to reduce the frequency of Tregs in tumors. Moreover, GVAX increased the frequency of the MDSC population in tumor leukocytes (Fig. 6c). On the other hand, FVAX increased the frequency of plasmacytoid DC (pDCs) levels in tumors (Supplementary Fig. 6), which have been shown to have immunosuppressive properties (47). Thus, vaccine alone increased TILs but also immunosuppressive cells.

Mice treated with GVAX or FVAX plus α PD-L1 showed significantly increased infiltration of total inflammatory CD45⁺ cells and T cells in tumors compared to mice treated with α PD-L1 ($p < 0.01$) or vaccine ($p < 0.001$) alone. Among them, CD8⁺ T cells were more abundant than CD4⁺ T cells (Fig. 6a and Supplementary Fig. 7a). When PD-L1 blockade

was combined with GVAX or FVAX, there was a moderate reduction in Treg as well as MDSC levels in the tumor (Figs. 6b&6c; and Supplementary Figs. 7b&7c). The addition of α 4-1BB to α PD-L1 increased significantly TILs relative to each agent alone, both CD8⁺ and CD4⁺. In addition, it markedly reduced Treg cells relative to each agent alone. Consequently, the combination of α 4-1BB plus α PD-L1 resulted in a marked increase in the CD8/Treg and CD4/Treg ratios, superior than each antibody alone. The combination of α PD-L1 and α 4-1BB with GVAX or FVAX yielded the highest frequency in TILs, both CD8⁺ and CD4⁺. Furthermore, the addition of vaccine did not increase Tregs when α PD-L1 and α 4-1BB were combined with GVAX or FVAX, thus resulting in the highest CD8/Treg and CD4/Treg ratios seen in this study. The triple combination also yielded the highest frequency of Ki67⁺ CD8⁺ T cells compared to that seen with the other combinations (Figs. 6d and Supplementary Fig. 7d). Combination treatment (either GVAX or FVAX plus α 4-1BB and α PD-L1) also increased granzyme B expression, suggesting an enhancement in cytolytic potential of effector T cells (Figs. 6d and Supplementary Fig. 7d). Increased phosphorylation of T-bet and Eomes was induced by α PD-L1 antibody alone, and addition of α 4-1BB to α PD-L1 produced only moderate further increases (Fig. 6e and 7e). Thus, the PD-L1 blockade induced TIL activation, and the combination of GVAX (or FVAX) and α 4-1BB treatment resulted in a further stimulation of the proliferative and cytolytic capability of TILs. Taken together, these changes could result in a more permissive environment for immune-mediated tumor rejection.

Combination therapy results in maximal stimulation of tumor-reactive CD8⁺ TILs

Given the significant changes in the tumor microenvironment, we next tested whether the functional capacity of tumor-reactive CD8⁺ TILs was increased following combination therapy. Tumors from the various treatment groups were freshly dissociated enzymatically, and total leukocytes isolated from these tumors were seeded in primary mixed co-cultures and stimulated with K^b-restricted peptides of folate receptor- α or mesothelin, two known ovarian tumor antigens expressed by ID8 cells. TIL cultures derived from mice treated with GVAX or FVAX showed minimally increased IFN- γ ⁺ and TNF- α ⁺ CD8⁺ TIL when stimulated with the FR- α (SSGHNECPV) and mesothelin (LSIFKHKL and LIWIPALL) peptides (Fig. 7a & b; and Supplementary Fig. 8). TIL cultures derived from mice treated with α PD-L1 alone showed modestly increased IFN- γ ⁺ and TNF- α ⁺ CD8⁺ TIL when stimulated with the peptides. TIL cultures derived from mice treated with GVAX (or FVAX) plus α PD-L1 (not shown) or α 4-1BB showed further increased IFN- γ ⁺ and TNF- α ⁺ CD8⁺ TIL when stimulated with the peptides. However, when mice were treated with α PD-L1 combined with GVAX (or FVAX) and α 4-1BB, TILs exhibited the highest numbers of IFN- γ ⁺ and TNF- α ⁺ CD8⁺ T cells ex vivo (Fig. 7a & b). Importantly, the increase in IFN- γ production by TILs specific to FR- α and mesothelin peptides appeared to be correlated with a decrease in tumor load (Figs. 3 & 5). The functional status of CD8⁺ TILs was corroborated by testing the phosphorylation status of key transcription factors. Triple combination therapy showed significant increases in pT-bet and pS6K levels, although only modest increase in pEomes (Fig. 7c).

Discussion

In this study, we demonstrate a critically important role of the PD-1 pathway in establishing an immune privilege site in the ovarian cancer microenvironment. Although tumors were infiltrated by T cells in very early stages of tumor orthotopic inoculation, a progressive reduction was noted in the frequency of CD8⁺ cells and the CD8/Treg cell ratio, until very few TILs were finally found in advanced tumors. Most of these were Tregs. The role of Tregs in mediating immune suppression and tumor growth has been previously established in ovarian cancer (24, 34). A parallel increase in MDSC and TAMs completed the picture of

a well-organized tumor immune suppressive environment. These findings are important in two ways. First, they indicate that lack of tumor immunogenicity is not intrinsic, but rather acquired, at least in ID8 ovarian tumors. Second, we found that such progressive establishment of immune suppression can be reversed by blocking the PD-1 pathway. Thus, PD-1 besides mediating TIL exhaustion, can facilitate a profound depletion of TILs. Importantly, this phenomenon was mediated by PD-L1, since PD-L1 blockade produced similar results, but not PD-L2.

PD-1 blockade restored the immune decline and led to expansion of TILs. An increase in the number of CD4⁺ and CD8⁺ cells was associated with increased CD8/Treg and CD4/Treg ratios. This was associated with a moderate clinical response, along with molecular evidence of functional activation of TILs. Reversal of decline was time-sensitive, since PD-1 blockade was more effective in earlier times, when more TILs were present in tumor microenvironment. Moreover, the therapeutic effect of blocking PD-1/PD-L1 correlated with increased numbers of polyfunctional ovarian tumor-antigen (mouse folate receptor- α and mesothelin) specific CD8⁺ T cells. Our study also provides further insight that tumor antigen-specific CD8⁺ T cells upregulate the key effector and memory precursor signaling molecules (T-bet, Eomesodermin, Akt, and S6 kinase) up on PD-1/PD-L1 blockade. This is in agreement with recent studies that described inhibition of signaling pathways by PD-1/PD-L1 mediated T cell exhaustion (48–53).

Given this observation, we theorized that expanding the pool of tumor reactive TILs through a whole tumor antigen vaccine (GVAX or FVAX) could be beneficial in more advanced time points of the disease. We found that in fact, although ineffective on its own, vaccine could double the efficacy of PD-L1 blockade. Importantly, vaccine inefficiency was not due to its inability to expand tumor reactive TILs. Rather, increase in TILs was accompanied by a similar (compensatory) increase in Treg and other immunosuppressive populations (such as MDSC or pDCs). Thus failure of vaccines was due to their failure to reprogram the tumor microenvironment, which continued to take advantage of homeostatic anti-inflammatory mechanisms such as Treg, MDSC and/or pDCs. Importantly, PD-L1 blockade again was able to break these homeostatic mechanisms, to establish tumor rejection. In addition, we found administration of agonistic 4-1BB stimulation and α -PD-L1 after vaccination produced maximal expansion of TILs and tumor rejection. We acknowledge that the dose of 4-1BB antibody (200ug) used was high for human translation and that in humans, 4-1BB agonistic Abs have resulted in liver toxicity (54). However, we believe that our studies still provide proof of principle for adding costimulation to checkpoint blockade as a beneficial therapeutic combination. Interestingly, similar benefit was also obtained by administering CpG, a TLR9 agonist, intraperitoneally.

In this model PD-L1 blockade was very effective while PD-L2 blockade was ineffective. This effect is likely due to the high expression level of PD-L1 in ID8 tumor cells as well as tumor-derived myeloid cells. Although α PD-L2 blockade resulted in moderately increased levels of TIL infiltration (CD45⁺, CD8⁺), proliferation (Ki67⁺CD8⁺) and signaling molecules (Eomes, Akt, and pS6 kinase), Treg and MDSC levels were not reduced after α PD-L2 blockade. In this regard, we previously noticed low level expression of PD-L2 in other types of tumor models (23). Hence the lack of response to α PD-L2 blockade may be due to low level expression of PD-L2, lacking sufficient endogenous response upon *in vivo* blocking PD-L2. Although our studies provide strong evidence of a role of PD-L1 in the syngeneic ovarian model, they do not allow us to discern the relative contribution of PD-L1 expressed by tumor cells versus tumor stroma or infiltrating leukocytes. Future studies using PD-L1 KO mice and bone marrow chimeras will be required to clarify this point. PD-L1 blockade overcame the upregulation of MDSCs and pDCs by GVAX and FVAX, respectively. Previous studies reported that FVAX was more effective in combination with

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| TIL | tumor-infiltrating lymphocytes |
| PD-1 | programmed death-1 |
| PD-L | programmed death-1 ligands |
| TAM | tumor associated macrophages |
| DC | dendritic cells |
| MDSC | myeloid derived suppressor cells |
| Tregs | Foxp3 ⁺ CD4 ⁺ CD25 ⁺ T cells |
| ID8-GVAX or ID8-FVAX | ID8 tumor cells transduced to express GM-CSF or Flt3-ligand |

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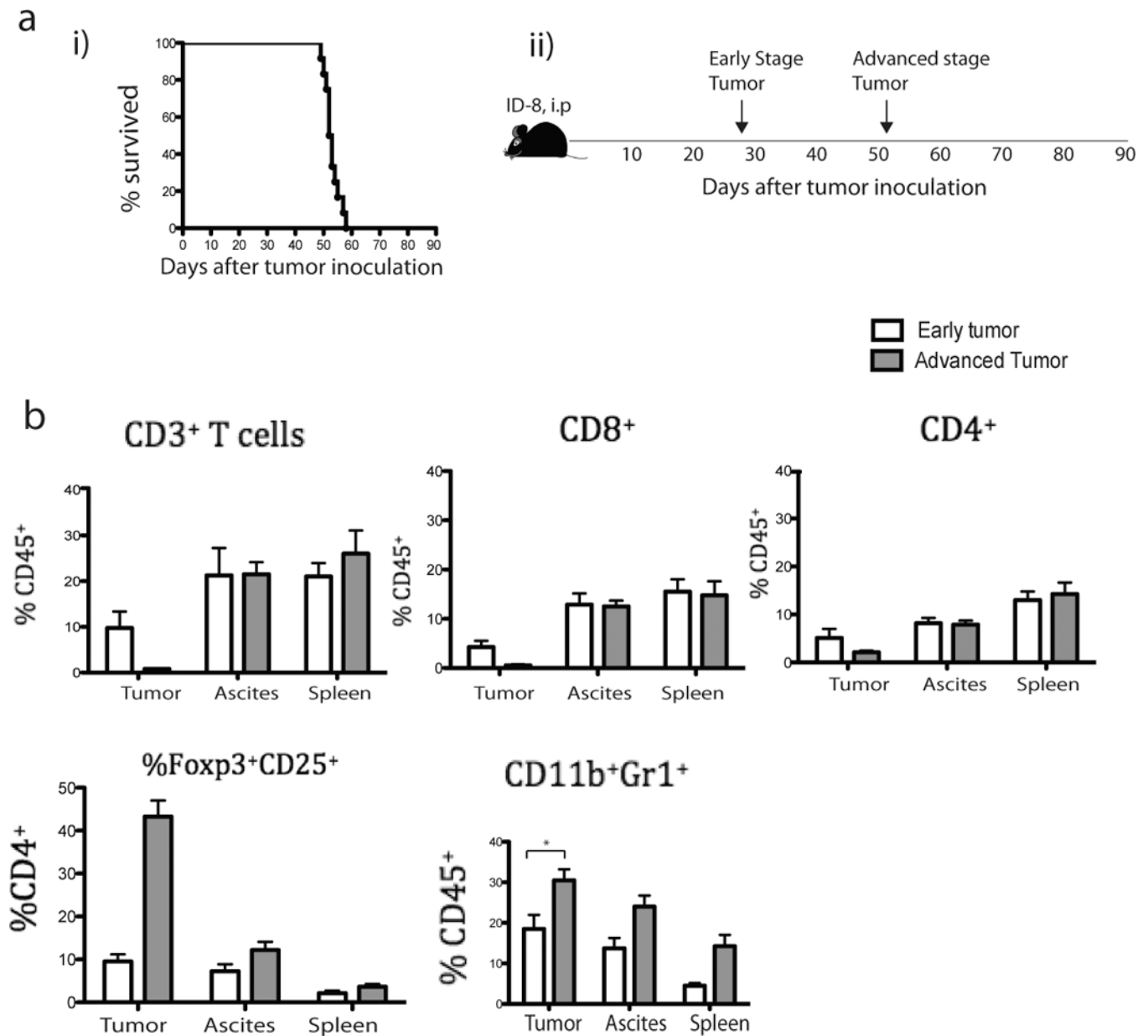


Figure 1. Accumulation of myeloid and T cell populations in ID8 tumor

(a) Six to 8 weeks-old mice (n=12) were inoculated i.p. with 5×10^6 ID8 tumor cells. The survival (i) and scheme (ii) are shown. (b) T (CD3⁺, CD8⁺, and CD4⁺) cells, Treg (CD4⁺CD25⁺Foxp3⁺) cells, and MDSC (CD11b⁺Gr1⁺) from tumor, ascites, spleen of ID8 tumor-bearing mice were isolated and counted (using 0.4% trypan blue stain) from 6 mice during early (4–5 weeks after tumor inoculation) or rest of the 6 mice during advanced (7–8 weeks after tumor inoculation) tumors. All cell subtypes were CD45⁺ gated. Bars represent mean \pm SEM.

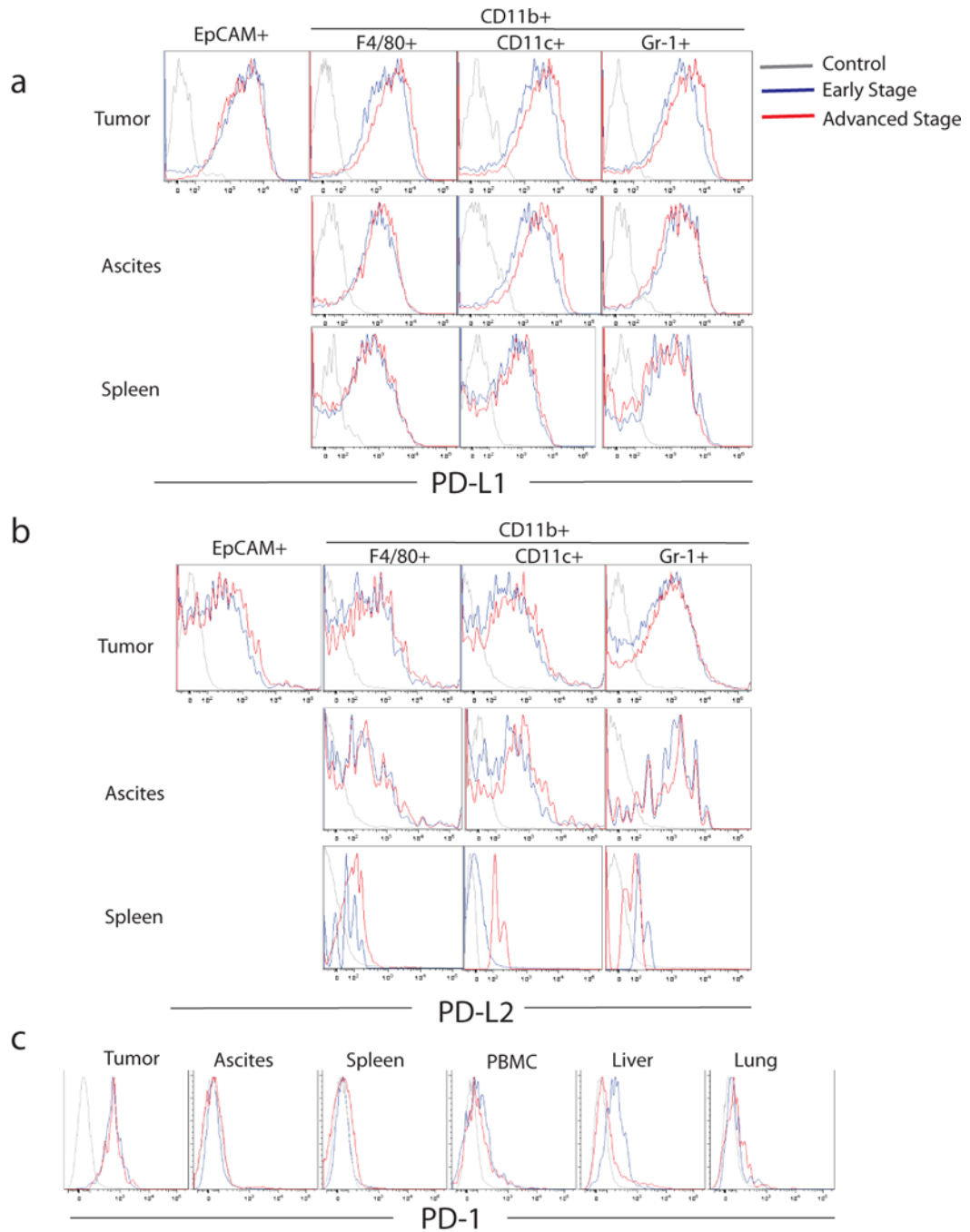


Figure 2. Expression of PD-L1, PD-L2 and PD-1 in ID8 mice

Mice were inoculated i.p. with 5×10^6 ID8 tumor cells ($n=12$), and their tumor, ascites, spleen, liver, lung and blood were harvested from half of the mice at early and the other half at advanced tumor. Tumor cells (EpCAM⁺), macrophages (CD45⁺CD11B⁺F4/80⁺), DCs (CD45⁺CD11c⁺) and MDSCs (CD45⁺CD11B⁺Gr1⁺) were isolated from the tumor, ascites and spleen. Histograms show PD-L1 (a) and PD-L2 (b) expression by ID-8 tumor cells as well as macrophages, DCs and MDSCs from tumor, ascites and spleen of ID-8 tumor-bearing mice. (c) PD-1 expression on CD8⁺ T cells from tumor, ascites, spleen, blood, liver and lung of ID-8 tumor-bearing mice. Results are from one of the 3 experiments.

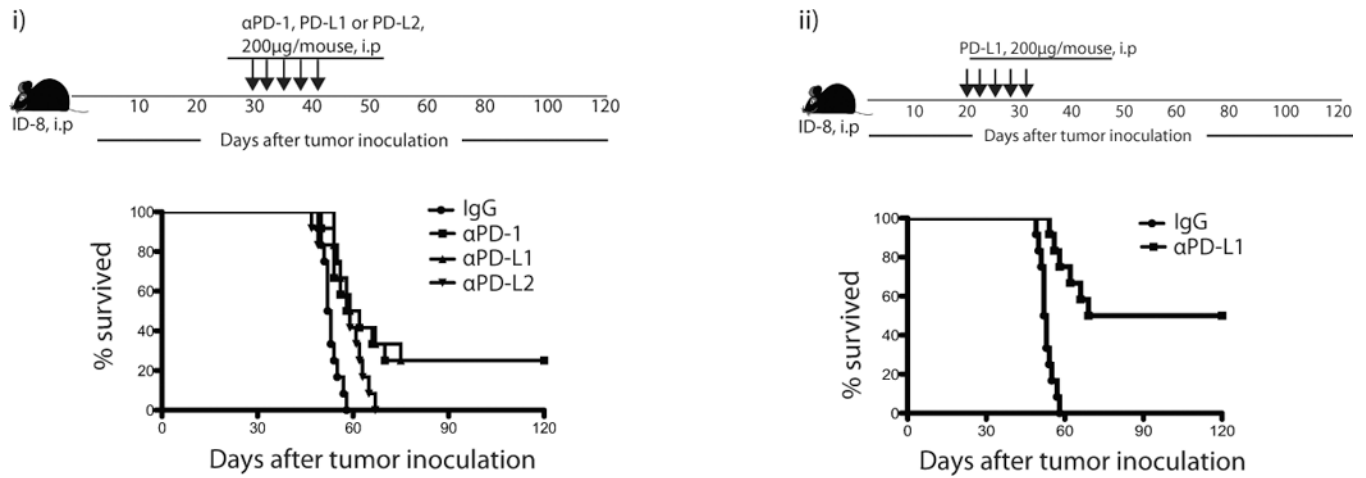


Figure 3. PD-1 or PD-L1 blockade causes regression of ID8 tumor

Mice were inoculated i.p. with 5×10^6 ID8 tumor cells (n=12). These mice were injected either therapeutically (starting at 30 days after tumor inoculation) or prophylactically (starting at 20 days after tumor inoculation) 5 times i.p. with α PD-1 (200 μ g), α PD-L1 (200 μ g), or α PD-L2 (200 μ g) blocking antibodies on alternate days either alone or in combination as indicated. The scheme and survival of therapeutic (i) and prophylactic models (ii) are shown. Results are from one of the 3 representative experiments.

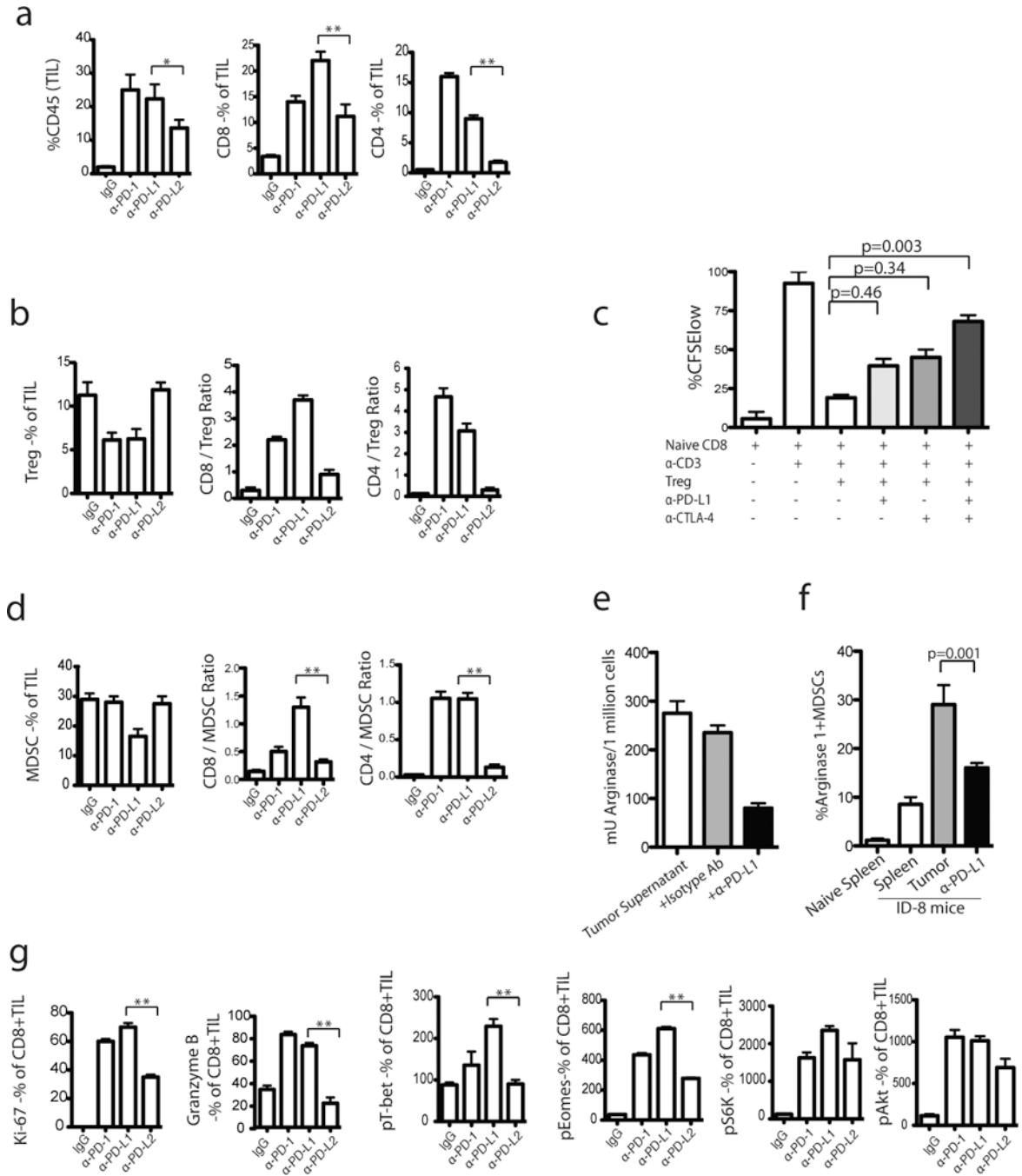


Figure 4. PD-1 or PD-L1 blockade increases immune activation of TILs in ID8 tumor
 One week following completion of blockade treatment, the TILs were harvested from regressing tumors and stained with various markers. Percentages of CD8⁺ and CD4⁺ TIL (CD45⁺) infiltration of total leucocytes (a) and the ratio of CD8⁺ T cells to Tregs (b) are shown in treated versus untreated groups. (c) Blocking PD-1/PD-L1 interaction reduced Treg-mediated suppression of CD8⁺ T cells *in vitro*. CFSE-labeled CD8⁺ T cells were co-cultured with syngeneic, αCD3-loaded DCs with or without Tregs and αPD-L1 or αCTLA-4 as indicated. CD8⁺ T cells and stimulator APCs were obtained from naive B6 mice. Tregs were obtained from ID8-tumor bearing mice. Treg-mediated suppression of proliferation of

naïve CD8⁺ T cells was noticed. Results from one of 3 experiments are shown. (d) The ratio of CD8⁺ T cells to MDSCs are shown. (e) CD8⁺ TILs from αPD-L1 treated mice were stained with arginase-1 (8C9 clone from Santa Cruz) and analyzed by flow cytometry. The CD11b⁺arginase-1⁺ MDSCs within CD45⁺ TILs are shown. (f) Tumor-derived MDSCs were plated at 1×10^6 /well in 24-well plates and stimulated with equal amount of tumor supernatants (from ID8 cells). Following stimulation, cells were added with αPD-L1 and then arginase I was analyzed after 24 h following washing with PBS and lysis buffer. (g) Percentage of Ki67⁺ and Granzyme B⁺ as well as MFI of pT-bet, pEomes, pS6, and pAkt expression by CD8⁺ TILs are shown. The results are the sum of three independent experiments with 8–10 mice per group.

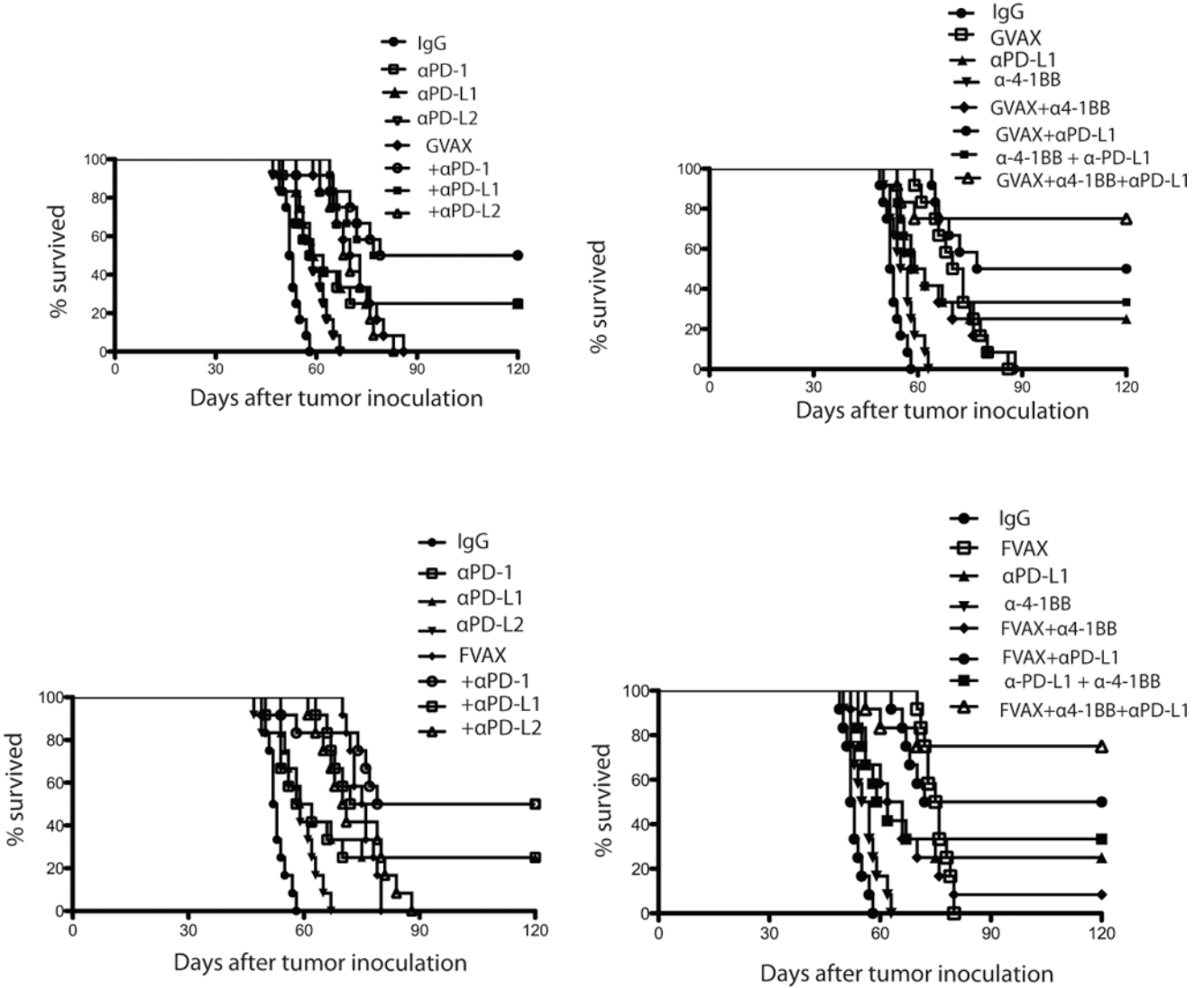
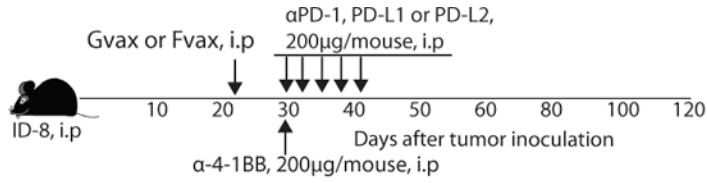


Figure 5. Synergistic effect of PD-1 blockade, GVAX or FVAX vaccination, and alpha-4-1BB costimulation on ovarian ID8 tumor rejection

(a) Three weeks after ID8 tumor inoculation, the mice were given i.p with 2×10^6 irradiated (150Gy) GVAX (top) or FVAX (bottom) once. A week later mice were injected 5 times i.p. with alpha-PD-1 (200ug), alpha-PD-L1 (200ug), alpha-PD-L2 (200ug) on alternate days or a single dose 200ug of alpha-4-1BB either alone or in combination as indicated. Results are from one of the 3 experiments.

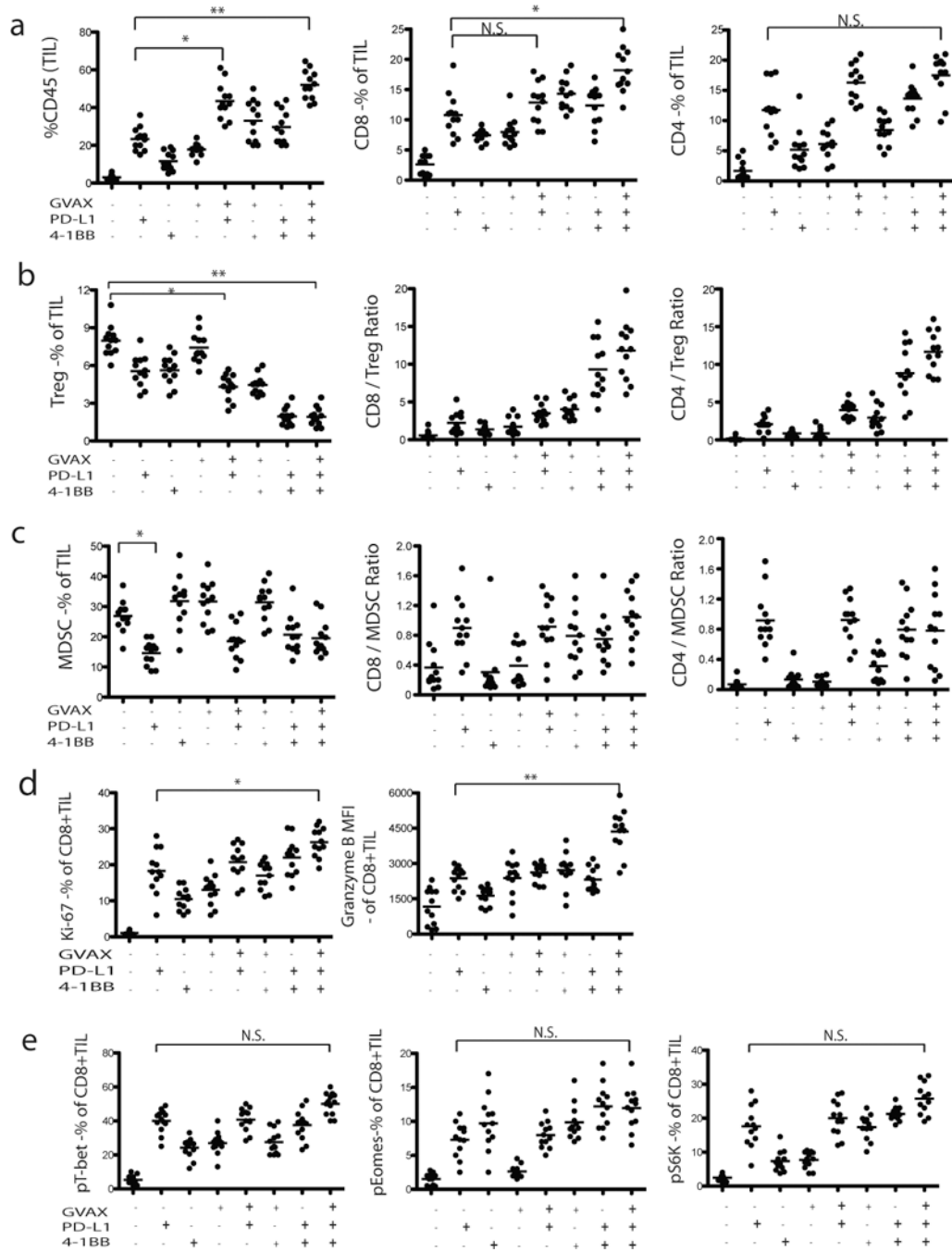


Figure 6. Synergistic effect of PD-1 blockade, GVAX vaccination, and α 4-1BB costimulation on immune activation

TILs from regressing tumors of ID8 mice were harvested a week following completion of treatment and stained for various markers. Percentages of CD8⁺ and CD4⁺ TIL (CD45⁺) infiltration of total leucocytes are shown in treated versus untreated groups. The ratio of CD8⁺ T cells to Tregs and MDSC are also shown. Percentages of Ki67⁺CD8⁺ and MFI of Granzyme B⁺CD8⁺ TIL (CD45⁺) infiltration of total leucocytes are shown in treated versus untreated groups. Mean intensity of pT-bet, pEomes, and p56K expression by CD8⁺ TILs is shown. Results from one of the 3 experiments are shown. Bars represent mean \pm SEM. *P 0.05; **P 0.01; ***P < 0.001.

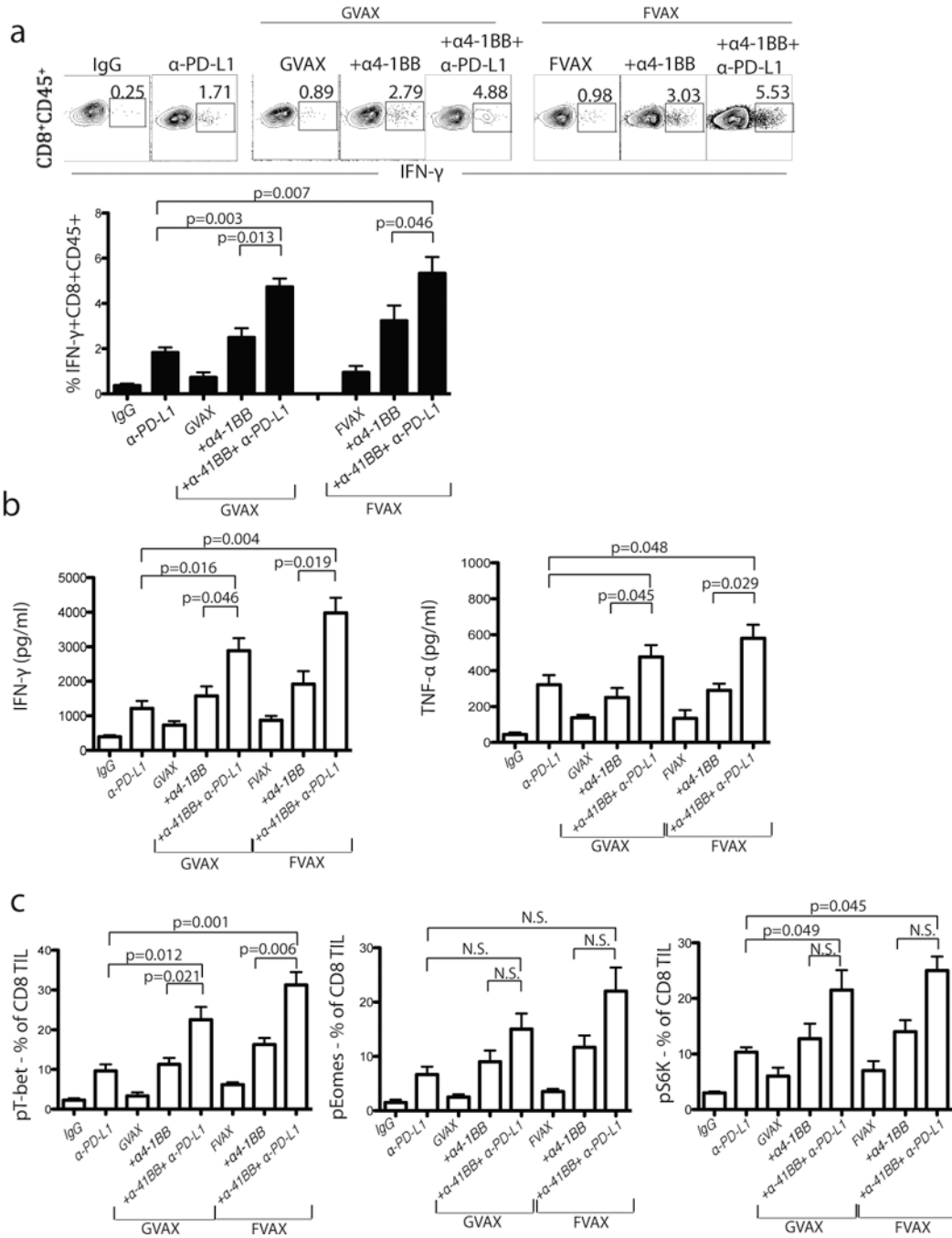


Figure 7. *In vivo* PD-L1 blockade increases ovarian tumor antigen (FR-α) specific inflammatory cytokine production by ID8 TILs

(a) Representative data and summary showing percentage of IFN-γ production by CD8⁺ TILs from treated mice as indicated. Following treatment with GVAX or FVAX and/or *in vivo* αPD-L1 and α-4-1BB antibody treatment, the TILs were harvested and stimulated *ex vivo* with FR-α peptide for 5 h at 37°C in the presence of brefeldin-A. For intracellular staining, permeabilized cells were stained for IFN-γ or phosphorylated form of transcription factors (T-bet, eomes and S6 kinase). (b) A week after completion of treatment, 2 × 10⁵ CD8⁺ T cells from 5–10 pooled tumor draining mediastinal lymph nodes were re-stimulated with 1 × 10⁵ FR-α peptide pulsed DCs for 36–48 h. Cytokine (IFN-γ and TNF-α)

production from culture supernatant was measured following *in vitro* culture using cytokine bead array (CBA) kit. (c) Flow cytometry analysis showing percentage of phosphorylated (p) T-bet, pEomes and pS6K expression by CD8⁺ TIL from treated mice. All analyses were performed using CD8⁺CD45⁺ TIL from treated mice. N.S. not significant.